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Bacitracin promotes osteogenic differentiation of human bone marrow mesenchymal stem cells by stimulating the bone morphogenetic protein-2/Smad axis

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ABSTRACT

Bacitracin, a widely used metallopeptide antibiotic, has been reported to be locally used in treating wounds without systemic adverse reactions. Our preliminary study showed that bacitracin might enhance the osteogenic differentiation of human bone marrow mesenchymal stem cells (HBMSCs). The present study investigated whether bacitracin affects the osteogenic differentiation of HBMSCs and the molecular mechanisms involved. The proliferation of HBMSCs in the presence of bacitracin was examined using a cell counting kit-8 (CCK-8) assay. The effects of bacitracin on the cell cycle and apoptosis of HBMSCs were observed using flow cytometry assay. Staining and quantitative assays for alkaline phosphatase (ALP) staining, collagen deposition (Sirius Red), and mineralization (Alizarin Red) were used to study osteogenic differentiation of HBMSCs. The expression of osteogenic differentiation markers was detected using quantitative reverse transcription polymerase chain reaction (RT-qPCR) analyses. The activation of related signaling pathways was examined using a luciferase reporter assay and western blotting. Bacitracin treatment increased osteogenic differentiation of HBMSCs without cytotoxicity and did not adversely affect cell cycle progression or apoptosis. The luciferase reporter assay showed that bacitracin activated the transcription of bone morphogenetic protein-2 (*BMP2*) gene, a key gene in the BMP2/Smad signaling axis. Western blotting indicated that this axis was markedly activated by bacitracin stimulation of osteogenesis. Moreover, the activation of Smad phosphorylation and osteogenic differentiation by bacitracin was inhibited by a transforming growth factor (TGF)-β/Smad inhibitor (LDN-193189 HCl) and small interfering RNA (siRNA) gene silencing (si-BMP2). In conclusion, our results suggest that bacitracin can promote osteogenesis of HBMSCs by activating the BMP2/Smad signaling axis.

1. Introduction

Infection is a common complication in orthopedics and often occurs in numerous types of orthopedic surgery. Bone infection can lead to a sequence of serious consequences such as protracted infected lesions, necrotic bone formation, loosened implants, and failed surgery, which require further treatments [1]. In addition to surgical removal of the infected tissue, use of antibiotics in bone-related infections is of vital importance [2]. Antibiotics are used intravenously to treat soft tissue or deep infections and may be impregnated into cement preparations or loaded into beads that are placed directly into the wound. As the infection is gradually controlled and the necrotic bone tissue is absorbed, the resulting osteolysis from the infection needs to be resolved [3]. However, common antibiotics currently used in the clinical treatment of bone-related infections, including β-lactams, cephalosporins, aminoglycosides, macrolides, and quinolones, do not effectively promote bone regeneration and differentiation. Gentamicin, an antibiotic commonly used for the clinical treatment of bone-related infections, inhibits the proliferation and viability of osteoblasts [4], while vancomycin inhibits osteoblast proliferation at certain concentrations [5]. It is important to find a drug that exhibits both antibacterial and osteogenic effects.

Human bone marrow mesenchymal stem cells (HBMSCs) are bone marrow-derived cells that play a key role in the renewal and regeneration of osteoblasts. HBMSCs can differentiate into bone-forming osteoblasts and have been shown to be a primary source of osteoprogenitor cells [6–8]. Moreover, HBMSCs can be used as bone graft materials to treat bone defects [9]. HBMSCs have been shown to differentiate into osteoblasts by treatment with dexamethasone, ascorbic acid, and β-glycerophosphate [10,11]. After a local infection is cleared,

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HBMSCs are activated and differentiate into osteoblasts to complete the repair of local bone dissolution. Failure of HBMSCs to completely repair the local bone defects caused by infection may lead to local osteoporosis and even pathological fractures [3]. Currently, drugs that function as bone absorption inhibitors by suppressing osteoclast activity are used for osteoporosis treatment [12], including vitamin D analogs and calcitonin [13]. However, these drugs cannot promote osteogenic differentiation and do not possess the necessary antibacterial properties to be used to treat orthopedic infection.

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor (TGF)-β superfamily that play a significant regulatory role in bone formation and are potent osteoblastic differentiation factors [14,15]. Among the members of the BMP subfamily, BMP-2 induces bone formation and differentiation *in vivo* and *in vitro* [16]. BMP-2 activates Smad1/5/9 proteins and mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38, and c-Jun N-terminal kinases (JNKs) [17–20]. Following activation, these signal transduction molecules increase the expression of Runt-related transcription factor 2 (Runx2) and core-binding factor alpha 1 (Cbfa1) [21–23].

Bacitracin is a widely used metallopeptide antibiotic produced by *Bacillus subtilis* and *B. licheniformis*. It strongly inhibits gram-positive bacteria and has a significant antagonistic effect on the development of *Staphylococcus aureus* resistance [24]. Bacitracin possesses a thiazoline ring formed by condensation of the Ile-1 carboxylate and contains a unique cyclic heptapeptide structure formed *via* an amide linkage between the side chain of Lys-6 and the C-terminus of Asn-12. This particular structure might be associated with the ability of bacitracin to resist protease degradation [25]. Partial use of bacitracin in wound treatments could reduce adverse systemic reactions such as renal dysfunction, which is the most common complication of bacitracin use [26]. In our preliminary study, we found that bacitracin also enhanced the osteogenic differentiation of HBMSCs. Thus, its excellent bioactivity and multi-functional properties suggest that bacitracin might be a suitable agent for the treatment of local bone infection and osteolysis.

Considering the important role of the BMP2/Smad signaling axis in osteoblast differentiation and bone formation, we hypothesized that this signaling axis mediates the characteristic effects of bacitracin in promoting the osteogenic differentiation of HBMSCs. To verify this hypothesis in the present study, we investigated the effects of different concentrations of bacitracin on the osteogenic differentiation of HBMSCs and the degree of activation of BMP2/Smad signaling.

2. Materials and methods

2.1. Reagents

Bacitracin, dexamethasone, ascorbic acid, β-glycerophosphate, 16-alkyl pyridine, and sodium phosphate were purchased from Sigma-Aldrich (MO, USA). Dexamethasone was dissolved in dimethyl sulfoxide (DMSO), while ascorbic acid and β-glycerophosphate were dissolved in phosphate-buffered saline (PBS). Fetal bovine serum (FBS) and trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA, 0.5%) were purchased from Gibco (USA). Alpha-modified Eagle medium (α-MEM) and penicillin/streptomycin solution were purchased from Hyclone (USA). The following antibodies used in the flow cytometry analysis and their isotype controls were purchased from BD Biosciences (USA): cluster of differentiation 90 (CD90), CD105, CD34, and CD45. The cell counting kit-8 (CCK-8) was purchased from Donjindo (Kumamoto, Japan).

The propidium iodide (PI)/RNase staining buffer and Annexin V Apoptosis Detection Kit were purchased from BD Biosciences. Alizarin red dye and Sirius red staining solutions were purchased from Servicebio (Wuhan, China). The alkaline phosphatase (ALP) color development kit, ALP quantitative kit, radioimmunoprecipitation assay (RIPA) lysis buffer, and phenylmethanesulfonyl fluoride (PMSF, 100×) were purchased from Beyotime Biotechnology (Nantong, China). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). The real-time polymerase chain reaction (qPCR) kit (SYBR® Premix EX Taq™) and cDNA synthesis kit) were purchased from TaKaRa (Japan). The protease inhibitor cocktail (100×) and bicinchoninic acid (BCA) protein assay kit were purchased from Thermo Fisher Scientific (USA).

The polyvinylidene fluoride membrane for western blotting and western chemiluminescent horseradish peroxidase (HRP) substrate (enhanced chemiluminescence, ECL) were purchased from Millipore (USA). The monoclonal antibodies against Smad1 (D59D7), Smad5 (D4G2), Smad4 (D3M6U), phosphorylated Smad 1/5 (P-Smad1/5, 41D10), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, D16H11), and the secondary HRP-conjugated anti-IgG antibody were purchased from Cell Signaling Technology (CST, USA). The monoclonal antibody against BMP-2 (18999-1-AP) was purchased from Proteintech (USA). The TGF-β/Smad inhibitor (LDN-193189 HCl) was purchased from Selleck Chemicals (Houston, TX, USA), while the Lipofectamine™ 3000 and Lipofectamine™ RNAiMAX transfection reagents were purchased from Invitrogen.

2.2. Cell culture

The HBMSCs were isolated and expanded as previously described [27]. The study was approved by the Ethical Committee of Shanghai Renji Hospital, Shanghai, China. Briefly, the bone marrow was diluted in α-MEM containing 10% (v/v) FBS. The suspension was seeded into T-25 cell culture flasks at 3.5 mL of bone marrow per flask. The cultures were maintained in an incubator at 37 °C in a humidified atmosphere of 5% CO_2, and the medium was replaced on the fourth day. The colony forming units were cultured with complete medium (α-MEM containing 10% FBS) until the cells were confluent. After digestion with trypsin-EDTA, the first generation cells were split into three T-25 cell culture flasks. The culture medium was changed every 2 days. Cells from passage 3 were used for experiments.

To identify the HBMSC phenotype, 1×10^6 third-passage adherent HBMSCs were washed in PBS and incubated with the following mouse anti-human monoclonal antibodies, PE-Cy™7-CD90 (positive), APC-CD105 (positive), PE-CD34 (negative), and PE-CD45 (negative). Isotype controls were prepared to distinguish non-specific from specific staining. The cells were incubated with antibodies for 45 min at room temperature. The cell suspensions were washed three times with PBS and then analyzed using a flow cytometer (FACSCalibur™, BD Biosciences, Franklin Lakes, NJ, USA; Fig. 1A).

2.3. Cytotoxicity, cell cycle, and cell apoptosis analysis

The cytotoxicity of bacitracin was assessed by performing a CCK-8 assay according to the manufacturer's instructions. In brief, HBMSCs (5.0×10^3 cells/well) were plated in 96-well plates with various concentrations of bacitracin (0, 0.1, 1, 10, or 100 μM) for 1, 3, or 5 days. Then, the cell culture medium was removed, and 100 μL of the CCK-8 solution was added to each well. After incubation for 2–3 h, the absorbance of the solution was read at 450 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader.

The effect of bacitracin on the HBMSC cell cycle was determined using flow cytometry with PI/RNase staining on culture day 1, 3, and 5. Briefly, at the 1-, 3-, and 5-day time points, HBMSCs were harvested and washed with PBS. Then, the cells were added to 75% ice-cold ethanol drop by drop while vortexing and fixed at −20 °C for 120 min in the dark. After fixation, the cells were washed twice to remove the ethanol, resuspended in 0.5 mL PI/RNase staining buffer, and then incubated for 15 min in the dark. The cell cycle proportions (G0, G1, S, and G2 M phases) were determined using a FACScan™ flow cytometer (BD Biosciences, USA).

Apoptosis was assessed using flow cytometry with double-staining for Annexin V-fluorescein isothiocyanate (FITC), which binds to